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A monoclonal antibody that causes the heterotrimeric G-protein G_o to release its βγ subunits

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Abstract Heterotrimeric (αβγ) guanine nucleotide binding proteins (G-proteins) dissociate into their constituent subunits in the course of signal transduction. Exposure of the G-protein G_o to the α_o-specific monoclonal antibody 3E7 results in recovery of α_o alone. We identified the 3E7 epitope as ERSKAIEKNL (positions 14–23) using synthetic peptides and phage display. G_o isolated with α_o-specific monoclonal antibodies MONO and 3C2 dissociates and releases its βγ subunits when exposed to 3E7. Exposure to 3E7, but not MONO or 3C2, results in the displacement of βγ from trimers, in the absence of added activators of G-proteins (GTPγS, $Mg^{2+}AlF^{4-}$). We propose that stable binding of 3E7 to α_o requires displacement of βγ and occurs in the absence of guanine nucleotide exchange.

Key words: Trimeric G-protein; Monoclonal antibody; Antibody-induced dissociation; Guanine nucleotide

1. Introduction

G-proteins (α (39–52 kDa), β (35–36 kDa) and γ (8–12 kDa)) transduce signals from cell surface receptors to their downstream effectors [1-4]. Their function is not limited to the cell surface: substantial evidence implicates G-proteins in intracellular membrane transport events [5-7]. G-proteins have been localized to subcellular domains such as caveolae and Golgi membranes [8,9]. Reconciling the prevailing view on G-protein-mediated signalling with the existence of intracellular pools of G-protein subunits involved in transport is a challenging cell biological problem. GTPγS and AlF^{4-}, commonly used activators of trimeric G-proteins, block intra-Golgi transport. How activation occurs in the course of G-protein-mediated transport is not known. It has been suggested that trimeric G-proteins may interfere with coatomer binding to intracellular membranes [7].

Activation of G-proteins by ligand-occupied receptors at the plasma membrane catalyzes the exchange of α-bound GDP for GTP, resulting in dissociation of the heterotrimer and the release of βγ. Both α and βγ can then regulate downstream effectors [10,11]. A recent study showing activation of an effector by a G-protein in the absence of GDP/GTP exchange or GTPase activity suggests that activation can occur by other means [12].

We have assessed the quaternary structure of the G-protein G_o, originally identified as the major pertussis toxin substrate in bovine brain [13-15], by using monoclonal antibodies (mAbs) which recognize trimeric and monomeric forms of α_o [16]. We mapped the epitope recognized by mAb 3E7 and localized it to the amino residues 14–23 of the α subunit. The structures of G_i and G_t, as determined by X-ray crystallography, confirm this region as a putative βγ-binding site in α subunits [16,17]. Exploiting the ability of two additional mAbs MONO and 3C2 to bind G_o trimers, we show here that exposure of G_o-antibody complexes to 3E7 results in the removal of βγ. We suggest that the release of βγ by 3E7 is best explained by 3E7 and βγ interacting with the same site on α_o. These results allow the conclusion that dissociation of trimeric G-proteins does not absolutely require GDP/GTP exchange, and could conceivably be accomplished by cellular proteins that interact with this segment of the G-protein α subunit.

2. Materials and methods

2.1. Preparation of membranes and immunoaffinity matrices

Bovine brain membranes and highly purified G-proteins were as previously described [18]. mAbs MONO, 3C2 and 3E7 (IgG_1 isotype) [18] were immobilized on CNBr-activated Sepharose-4B beads by covalent coupling methods described by the manufacturer (Pharmacia) or bound to protein A-Sepharose beads and covalently cross-linked [19].

2.2. Biochemical analysis

G-proteins were extracted from bovine brain membranes in cold Nonidet P-40 lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.3, and 5 mM $MgCl_2$) supplemented with 50 mM NaCl, 1 mM PMSF and 0.1% Lubrol for 2 h at 4°C. Lysates were clarified by centrifugation at 14000 rpm for 20 min prior to immunoprecipitations, which were typically carried out for 90 min at 4°C. Immune complexes were isolated, washed with cold NET buffer (0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) and prepared for SDS-PAGE [20]. Polypeptides were visualized by silver staining [21]. In some experiments, Nonidet P-40 lysates of bovine brain membrane preparations were applied to MONO or 3C2 affinity matrices at 4°C to immobilize G_o. The G_o-mAb isolated complex was washed with Nonidet P-40 lysis buffer and then equilibrated with buffer A (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 2 mM $MgSO_4$, 100 mM NaCl, 0.05% (w/v) Lubrol-PX (Sigma)). Specific elution of βγ was carried out with buffer A containing guanosine 5′-3-*O*-(thio)triphosphate (GTPγS) or 3E7 as described in the figure legends.

2.3. Peptide synthesis and analysis

Peptides were synthesized on a multiple peptide synthesizer (model 350; Advanced Chemtech, Louisville, KY), using conventional Fmoc chemistry. Peptides were dissolved in 0.1×phosphate buffered saline (PBS) and filtered through 0.22 mm filters [22]. Peptide concentrations were determined by absorbance at 275.5 nm and stored in 0.1 M borate buffer, pH 10 approximately at 1 mg/ml [23]. ELISAs and peptide competition assays were performed using the standard ELISA procedure and are as described in the figure legends [18]. The fd-tet M13 phage peptide library was prepared from a vector containing the fd-tet phage and the tetracycline gene [24]. Immunoprecipitations from the fd-tet 15mer library containing 6×10^{10} phage units in 0.5 ml of Nonidet P-40 lysis mix were carried out with 3E7. Immune complexes eluted with glycine HCl, pH 2.2 were used to infect K91 (kanamycin resistant) cells at room temperature. Phage particles were isolated from infected cells selected in the presence of 20 μg/ml tetra-

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cycline. The 3E7-selected phage particles were further amplified through two more rounds of immunoprecipitations, infections and phage isolation steps. Single stranded DNA particles were isolated from the final amplified phage library and sequenced [25].

3. Results

3.1. Identification of the 3E7 epitope

Two independent procedures were used to map the epitope recognized by the α_o-specific mAb 3E7. Peptides spanning residues 1–90 of the amino terminus of α_o, each 10 amino acids long, were synthesized using standard Fmoc chemistry. The sequences of the first six peptides are shown in Fig. 1A. The peptides were each tested for reactivity with 3E7 by ELISA. One peptide, Ala^{11}–Glu^{25} of sequence AALERSKAIEKNLKE, was substantially more reactive with 3E7 than any of the other peptides tested (Fig. 1B). In an ELISA assay using immobilized G-proteins on the plate, Ala^{11}–Glu^{25} was the only peptide which competed significantly with 3E7 for binding to the immobilized G-proteins (Fig. 1C). From these results, the epitope for 3E7 was localized to residues 11–25 in the amino terminus of α_o.

The 3E7 epitope was confirmed and further narrowed down by using an M13 phage display peptide library [24] to identify sequences of random peptides recognized by 3E7. The motifs obtained from sequencing single stranded DNA isolated from affinity-purified phage, as described in Section 2, are shown in Fig. 1D. The motif ERxxAxxxNL was common to 80% of the clones tested. Importantly, this completely independent strategy identified a segment of α_o wholly contained in the Ala^{11}–Glu^{25} peptide. The arginine at position 21 was present in all phage clones tested, though absent in the published sequence of α_o. The ability of 3E7 to discriminate between various α subunits was shown previously [18] and may be due to the presence of the alanine at position 18 (Ala^{18}), although we have not investigated this further. We propose that the epitope recognized by 3E7 on α_o is ^{14}ERSKAIEKNL^{23}.

3.2. 3E7 induces dissociation of G_o ($\alpha_o\beta\gamma$) trimers

The epitope recognized by 3E7 resides in a region of α shown to bind $\beta\gamma$ in other G-proteins [16,17]. In immunoprecipitations, mAbs MONO and 3C2 immunoprecipitate stable heterotrimeric complexes of α_o, β and γ subunits from preparations of G-proteins ([18,26]; see Fig. 2A, antibodies MONO and 3C2)[1]. In contrast, 3E7 immunoprecipitates α_o free of any associated $\beta\gamma$ (Fig. 2A, antibody 3E7). 3E7 may recognize only α subunits that have spontaneously released their $\beta\gamma$ subunits. Since $\alpha\beta\gamma$ are in equilibrium (K_d of α_o for binding $\beta\gamma$ was reported as 340–390 nM [27], but see [26]), 3E7 could potentially bind free α during trimer dissociation and by virtue of its high affinity prevent $\beta\gamma$ reassociation. Alternatively, 3E7 might interact with α_o by first inducing the release of $\beta\gamma$ from the trimeric complex. Therefore trimers immobilized on 3C2 matrices were exposed to 3E7 in solution. G_o was first collected from solubilized brain membrane extracts with 3C2 (Fig. 2B, 1st antibody 3C2). The $\beta\gamma$ subunits were co-immunoprecipitated by virtue of their association with α. Incubation of G_o with 3E7 resulted in the dissociation of all $\beta\gamma$ subunits (Fig. 2B, 1st antibody 3C2, 2nd antibody 3E7). The $\beta\gamma$ subunits were released into the supernatant, as revealed by immunoblot analysis with polyclonal serum reactive with α_o, β and γ subunits (Fig. 2C, 1st antibody 3C2, 2nd antibody 3E7). No loss of α from the 3C2-G_o complex was detected (Fig. 2C; 1st antibody 3C2, 2nd antibody 3E7). As control, a purified α_o/α_i mixture is shown in both figures (Fig. 2B,C, Pur G). Previous reports have shown that α_o-GDP has a high affinity for $\beta\gamma$ when bound to MONO or 3C2, such that spontaneous dissociation of the trimer is not observed [28]. It is therefore likely that it is the binding of 3E7 to its epitope that displaces $\beta\gamma$ from G_o.

We examined the stability of the trimer in the presence of two mAbs interacting simultaneously with the α subunit. We exposed Nonidet P-40 detergent lysates prepared from brain membranes to 3E7 and then scored for the effect on G_o seen by MONO or 3C2. Lysates were first incubated with 3E7 (Fig. 2D, 1st antibody, 3E7) and following removal of 3E7 immune complexes, residual G-protein subunits were immunoprecipitated with MONO (Fig. 2D, 1st antibody 3E7, 2nd antibody MONO) or with 3C2 (Fig. 2D, 1st antibody 3E7, 2nd antibody 3C2). Under these experimental conditions, no $\beta\gamma$ subunits were recovered with MONO or 3C2 (Fig. 2D). Both MONO (or 3C2) and 3E7 can interact simultaneously with α_o. 3E7 readily precipitates free α_o (generated by treatment of lysates with GTPγS), indicating that the presence of $\beta\gamma$ is not necessary for the antibody to bind α_o [18]. Exhaustive immunoprecipitation of lysates with 3E7 removes all α subunits reactive with MONO or 3C2 (Fig. 2E, see MONO or 3C2 following three sequential 3E7 immunoprecipitations). While it is possible that 3E7 binds free α and subsequently shifts the equilibrium such that only free αs are available to interact with MONO and 3C2, we favor the interpretation that association of 3E7 with α alters the interaction of α_o with $\beta\gamma$ and results in dissociation of the trimeric complex.

3.3. Comparison of dissociation of G-proteins by 3E7, with GTPγS

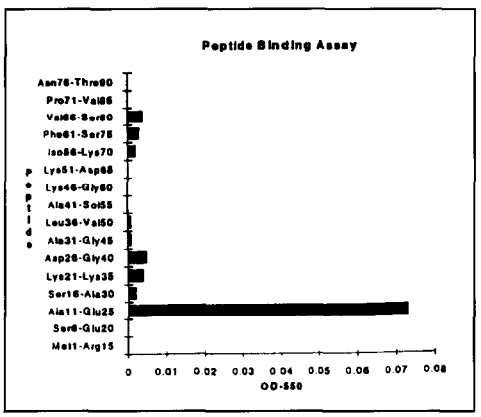
The efficiency of dissociation induced by 3E7 was compared with that of the nonhydrolyzable GTP analogue, GTPγS. Similar results were obtained with $MgAlF^{4-}$ (data not shown). G_o was collected from Nonidet P-40 detergent lysates with MONO-beads as described in the figure legend (Fig. 3A, MONO-isolated G_o). G_o was washed extensively and then treated with either GTPγS, 3E7 or with 3C2. As expected, GTPγS induced dissociation of G_o and caused the release of all $\beta\gamma$ subunits (Fig. 3A, GTPγS treatment). 3E7 induced release of the majority of the $\beta\gamma$ subunits in the MONO-isolate (Fig. 3A, 3E7 treatment). Note the appearance of the 3E7 light chain in the immunoprecipitate (Fig. 3A, 3E7 treatment), as indicator that α, immobilized on MONO, can interact simultaneously with 3E7. Eluants from a MONO-protein A-Sepharose column treated with either 3E7 or GTPγS showed near identical elution profiles (Fig. 3B). Exposure to 3C2 resulted in release of the trimer from the MONO-isolate (Fig. 3A, 3C2 treatment). We ascribe this to competition between the mAbs for their closely spaced epitopes, such that excess 3C2 (residues 80–145, 100–103 in particular [18]) in solution displaces G_o from MONO (residues 80–145 [18]; see Fig. 2C, 1st antibody MONO, 2nd antibody 3C2). We conclude that like GTPγS, 3E7 treatment of G_o, results in the physical separation of α and $\beta\gamma$ subunits.

[1]The specificity of the antibodies for α_o, and the assignment of α and β proteins has been published [18]. The γ subunits are not visualized under these electrophoretic conditions but can be readily seen on Tris/tricine gels (Rehm, Bogyo and Ploegh, unpublished data).

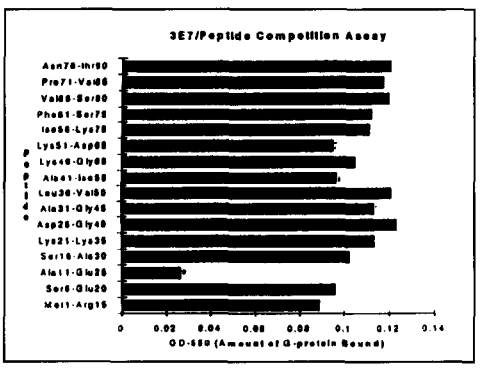
A

1. Met[1] - Arg[15] MGCTLSAEERAALER
2. Ser[6] - Glu[20] SAEERAALERSKAIE
3. Ala[11] - Glu[25] AALERSKAIEKNLKE
4. Ser[16] - Ala[30] SKAIEKNLKEDGISA
5. Lys[21] - Lys[35] KNLKEDGISAAKDVK
6. Asp[26] - Gly[40] DGISAAKDVKLLLLG

B



C



D

#	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	Residue Number
			s, k	E	R	w, n	m, p g	A	L	h, g	R	N	L			Phage Display
αo	A	A	L	**E***	**R***	S	K	**A**	I	E	K	**N***	**L***	K	E	
αx	E	A	A	*R*	**R***	S	R	*R*	I	D	R	*H*	**L***	R	S	
αi1	A	A	V	**E***	**R***	S	K	M	I	D	R	**N***	**L***	R	E	
αi2	A	A	V	**E***	**R***	S	K	M	I	D	R	**N***	**L***	R	E	
αi3	A	A	V	**E***	**R***	S	K	M	I	D	R	**N***	**L***	R	E	
αt1	-	-	-	-	*H*	S	R	*E*	L	E	K	*K*	**L***	K	E	
αt2	E	L	A	*K*	**R***	S	K	*E*	L	E	K	*K*	**L***	Q	E	
αs	A	Q	R	**E**	*A*	N	K	*K*	I	E	K	*Q*	**L***	Q	K	

Fig. 1. Epitope on α_o recognized by 3E7. A: Six of 16 overlapping 15mer peptides synthesized from α_o amino acids 1–90 are shown. B: 15mer peptides were plated at 0.1 μg/ml on ELISA plates and incubated with 50 μl of 3E7 hybridoma supernatant. The absence of bars for some peptides indicates no significant signal above background. C: 0.5 μg of purified G_o/G_i mixtures were immobilized on ELISA plates and then exposed to 50 μl of a mixture containing 3E7 hybridoma supernatant and 0.25 μg of each peptide. All ELISA experiments were done in triplicate and the average OD_{550} recorded. D: The proposed motif recognized by 3E7 based on phage display is given at the top. Conserved residues present in 10 phage clones used to construct the motif are capitalized. In bold are those residues found in α_o. Amino acids found in the 10 clones at other positions are in lower case. The table below the consensus motif lists the different α isotype sequences. An exact match of the motif with any other α subunit sequence with the motif is given in bold and an asterisk indicates residues shared with α_o. The residues in italics (positions 14, 15, 18 and 22) are substitutions that may preclude reactivity with 3E7. The residues occurring at positions 19 and 21 of the phage display motif occur as conservative substitutions in α_o (Leu-Ile and Arg-Lys, respectively). We obtain a match of 8/11 residues for the 3E7 motif with α_o if these conservative substitutions are included. The sequences are bovine α_o [33], rat α_x [34], rat [35], α_{i2} [36], α_{i3} [37], bovine α_{t1}, α_{t2}, rat α_s [37].

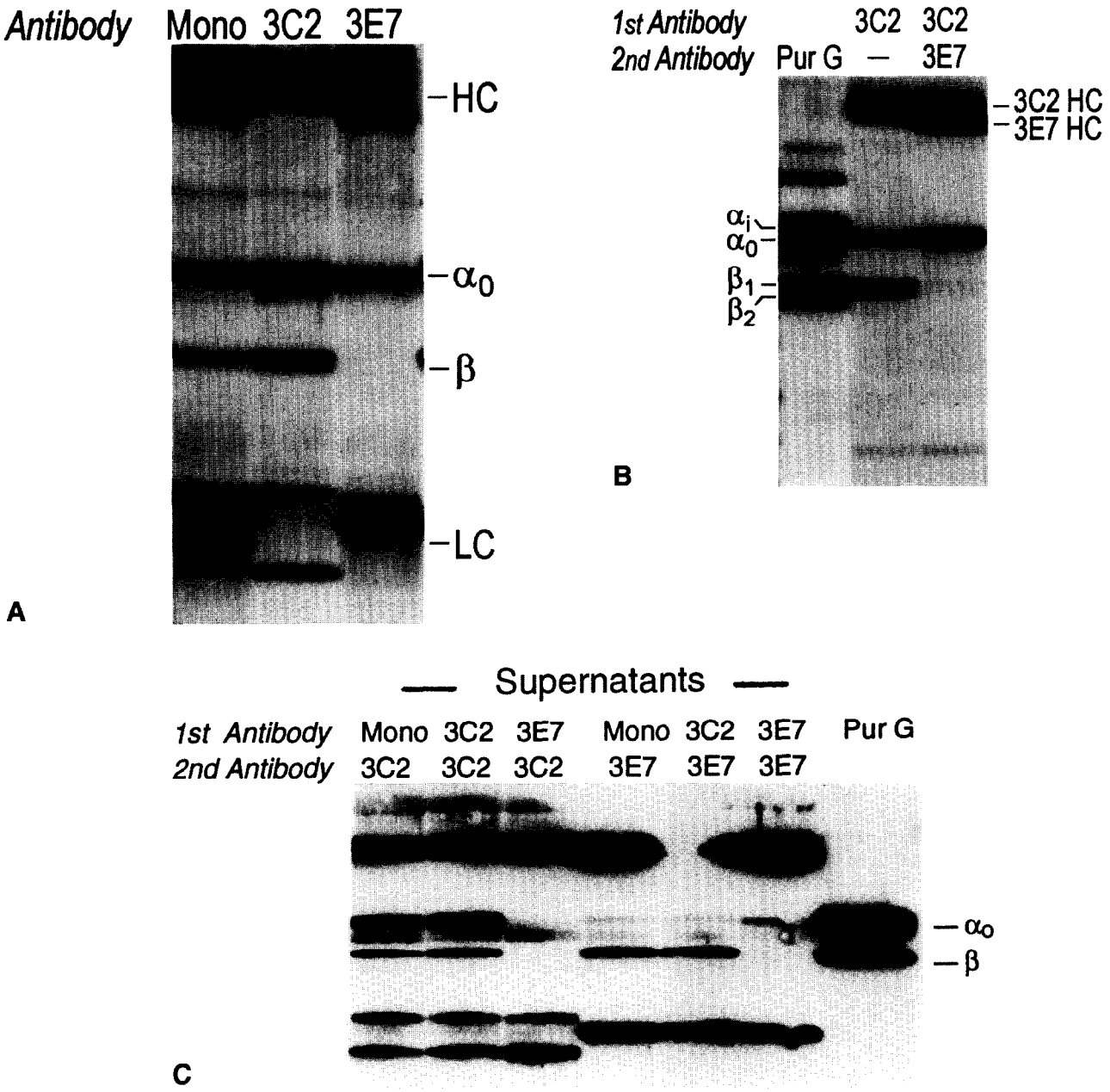
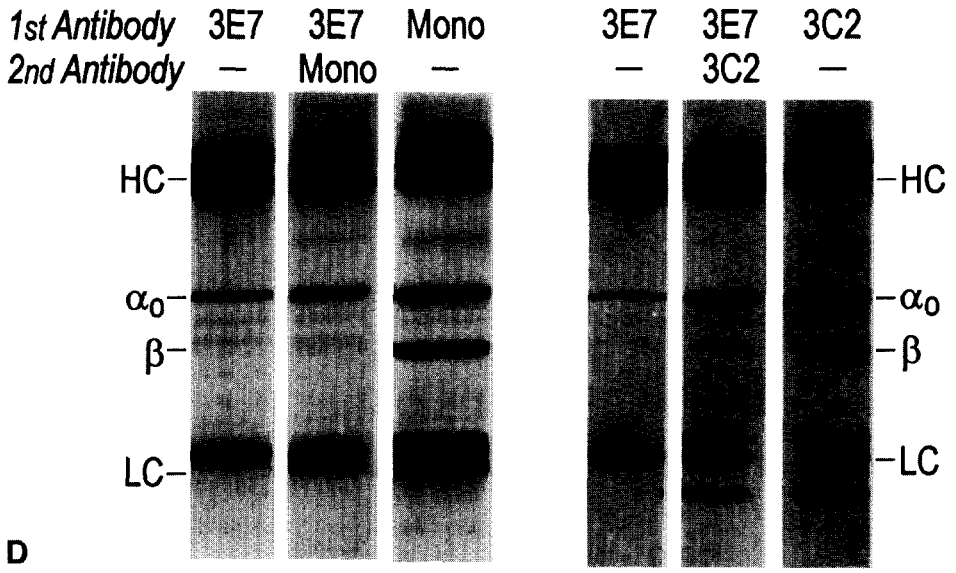


Fig. 2. G_o-antibody interactions. A: Non-denaturing Nonidet P-40 lysates of bovine brain membranes were immunoprecipitated with MONO, 3C2 and 3E7 and the immune complexes collected on *Staph. A*. Eluted samples were loaded on a 10% SDS-polyacrylamide gel and the polypeptide chains visualized by silver staining. The positions of immunoglobulin heavy (HC) and light (LC) chains, and the G-protein subunits, α_o (39 kDa) and β (refers to β1 (36 kDa) and β2 (35 kDa)), are indicated on the right of this and subsequent figures. B: Nonidet P-40 lysates of bovine brain membranes were immunoprecipitated with 3C2 for 60 min at 4°C and the immune complexes washed once with NET buffer. The isolated complex was then incubated with excess 3E7 (at 1 mg/ml) for an additional 60 min at 4°C. Input G-protein for each precipitation was 180 μg. Heptylamine-Sepharose purified G-proteins (Pur G), direct precipitation with 3C2 (1st antibody 3C2), 3C2-reactive G_o treated with 3E7 (1st antibody 3C2, 2nd antibody 3E7). The G-protein α_i (41 kDa) is indicated. C: Immunoblot analysis of the supernatants. Blots were probed with a bovine G-protein polyclonal serum reactive against α, β and γ subunits. D: Lysates were depleted of 3E7-reactive material for 60 min at 4°C and the 3E7-immune complexes collected on *Staph. A*. The resulting supernatant was immunoprecipitated with MONO or with 3C2. Direct precipitation with 3E7 (1st antibody 3E7), MONO precipitation after 3E7 depletion (1st antibody 3E7, 2nd antibody MONO) or directly (1st antibody MONO), 3C2 precipitation after 3E7 depletion (1st antibody 3E7, 2nd antibody 3C2), or directly (1st antibody 3C2). E: Membrane lysates were immunoprecipitated overnight with 3E7 and then through two subsequent rounds of precipitations with the same antibody. The 3E7-depleted lysate was then immunoprecipitated with MONO or with 3C2.

4. Discussion

We have shown here that mAb 3E7 interacts with G-proteins via residues 14–23 on the α subunit. Trimers of G_o challenged with 3E7 in solution release their βγ subunits, in the absence of guanine nucleotide exchange. Since GTP was not added to the incubations, and since purified G-proteins occur exclusively in the GDP-liganded state, GTPase activity cannot play a role in the dissociation of βγ observed here. An anti-transducin mAb 4A was previously described whose effects are similar to those of 3E7 [29-31]. The affinity of α_t for



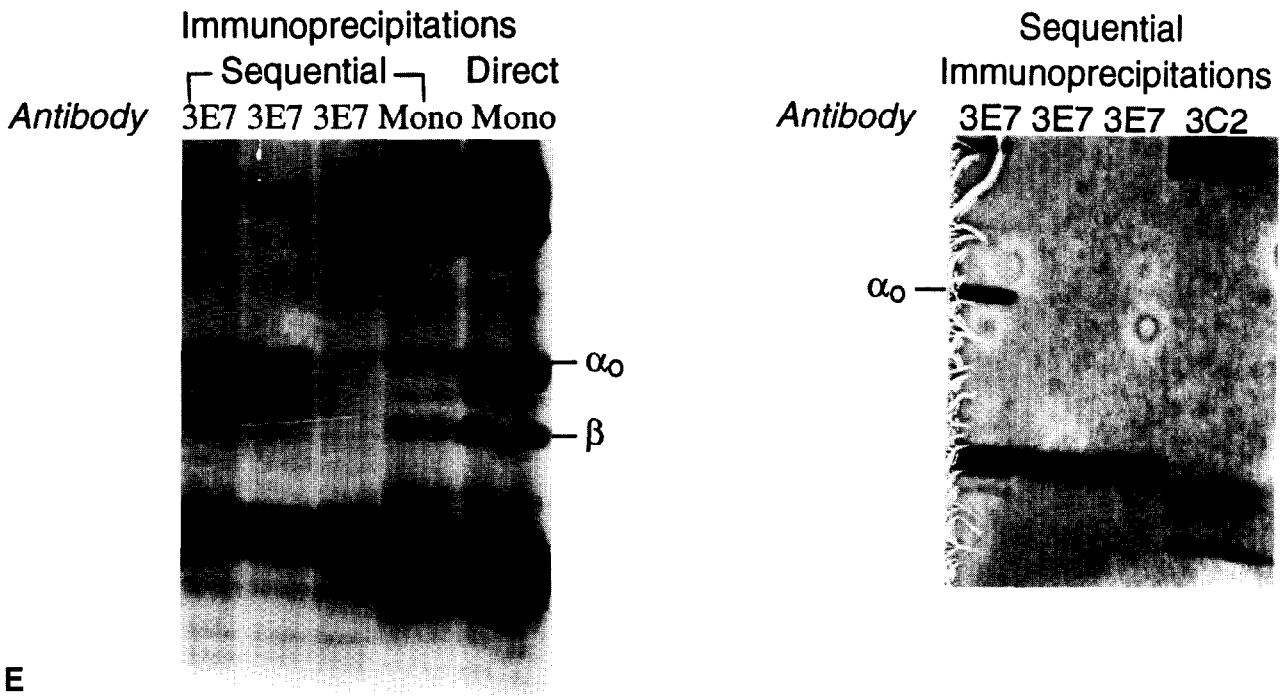


Fig. 2 (*continued*).

βγ is known to be substantially reduced when compared to other G-proteins, and it is the one G-protein for which dissociation in response to GTP (as opposed to GTPγS) has been shown [39]. The ability to raise antibodies of the type exemplified by 3E7 and 4A suggest that in a sizable proportion of the G-proteins in the immunogen, the respective epitopes must have been accessible. These observations are consistent with a dynamic interaction between α and βγ.

It could be argued that since α and βγ are in rapid equilibrium, 3E7 binds to free α in an irreversible manner and this generates free βγ subunits (see Fig. 4, model IIA). Formally, we cannot discriminate between this possibility and that of antibody-induced dissociation in some of our experiments (Fig. 4, model IIB). However, our biochemical data (Figs. 2 and 3) show that 3E7 can bind to G_o captured as stable trimers by MONO and 3C2, and in doing so displace their βγ subunits (Fig. 4, model I). From this it may be inferred that binding of 3E7 and βγ is non-equivalent. This is not altogether unexpected, because βγ interacts with α subunits, at least in the case of α_t and α_i in regions other than the N-terminus as well [16,17]. Since 3E7 does not readily dissociate from α, it eventually displaces all the βγ subunits bound to α. Binding of 3E7 may thus exclude rebinding of βγ. The 3E7 epitope does lie within the putative βγ binding region.

How does 3E7 release βγ? We consider two possibilities summarized in Fig. 4. to explain the observed displacement of βγ by 3E7. In the first possibility, 3E7 binds to a site that is adjacent to the region of α that contacts βγ. By binding of

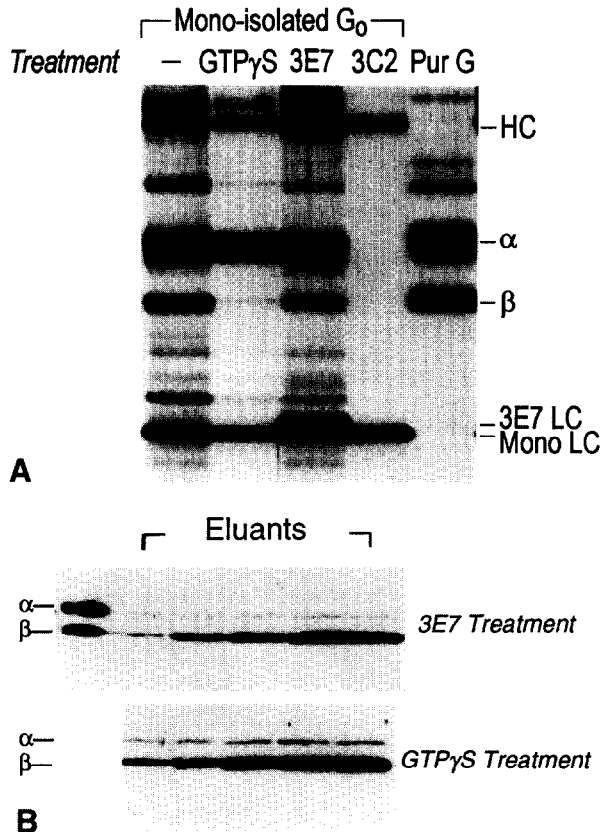


Fig. 3. Comparison of βγ release induced by 3E7, with GTPγ S. A: Nonidet P-40 lysates of bovine brain membranes were immunoprecipitated with MONO cross-linked to CNBr-activated Sepharose beads (MONO-beads). The MONO-isolated G_o (no treatment) was washed as described in the methods and then treated with either 0.5 mM GTPγS, 3E7 or 3C2 in buffer A. 3 μg of heptylamine-Sepharose purified G-proteins were loaded as control (Pur G). α indicates α_o (39 kDa), and α_i (41 kDa), present in the Pur G lane. B: Eluted fractions from an independent experiment are shown. G-proteins isolated on MONO-Sepharose beads were extensively washed as described and then eluted with 50 μM GTPγS or 100 μg/ml of 3E7.

3E7, the conformation of the βγ-interacting segment of α is altered such that dissociation is favored. In this scenario, the antibody would bind to the trimer, affect the βγ-binding segment, and lead to loss of βγ by favoring subsequent dissociation (Fig. 4, model IIB). There is evidence for a βγ interaction site proximal to the 3E7 epitope that we have identified [32]. In the second possibility, the G_o trimer exists in at least two states (Fig. 4, model IIC). In one of these alternative states, the interactions with βγ in the N-terminal region are relaxed to expose the site on α recognized by the antibody. The βγs remain attached to α via interactions with other segments of α. Upon binding of 3E7 to this newly exposed region, rebinding of βγ to that segment of α would be precluded and the complex of 3E7 and α_o would resolve into 3E7-α_o and free βγ. The conformation of α_o which is captured by 3E7 must be distinct from that seen in most of the naturally occurring trimers (which retain their βγs) and may well be distinct from that imposed by the binding of GTP. We have at present no tools to assess this possibility.

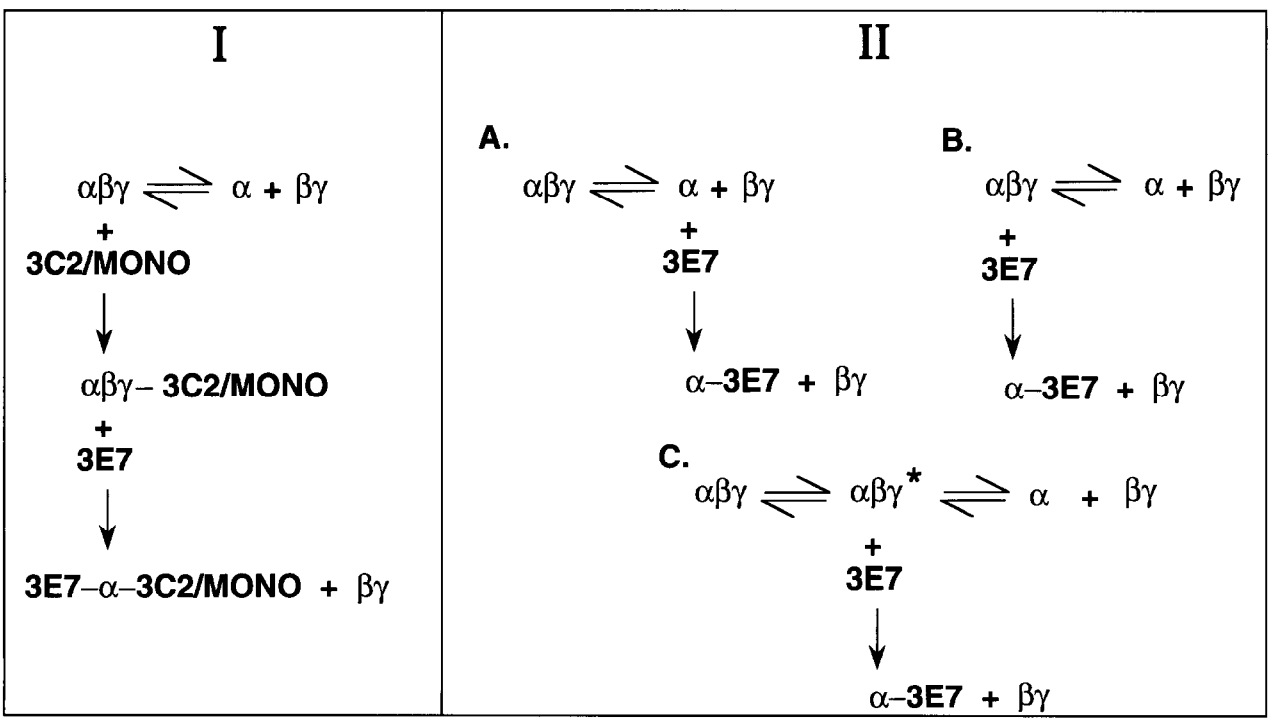


Fig. 4. Modes of interaction of 3E7 with G_o. In model I, the interaction of 3E7 with immuno-isolated G_o is shown. Binding of 3C2 or MONO to G_o shifts the equilibrium in favor of the trimer. Binding of 3E7 to the G_o-antibody complex induces the release of βγ subunits. In model II A, βγ is released as a consequence of 3E7 binding to the small pool of free α subunits available at equilibrium. In model II B, free βγ is generated by 3E7 binding to its epitope on α_o and subsequently displacing βγ subunits from the trimer. In model II C, a transition state of the trimer exists (αβγ*) in which the epitope for 3E7 is exposed. In this model, αβγ represents a closed state in which the epitope for 3E7 on α_o is masked.

This report shows a ligand in the form of a mAb that can induce dissociation of a G-protein trimer in the absence of GDP/GTP exchange. The mAb binds to a site on α not directly involved in guanine nucleotide binding. Using recombinant α_t and mutant α_t, the mutant form locked in the GDP-bound form was constitutively active toward the transducin effector molecule cyclic guanosine monophosphate phosphodiesterase in the absence of GDP/GTP exchange and GTPase activity [12].

The conventional view holds that ligand-activated receptors promote nucleotide exchange as well as dissociation of βγ subunits. Given the known biological activities of isolated βγ subunits, their levels are likely to be strictly controlled. Is activation of heptahelical receptors the sole means by which levels of βγ are controlled? An imbalance in the synthesis of βγ versus α subunits, or differential turnover of αs released upon activation, compared with the βγs thus released, could also contribute to the generation and maintenance of free βγs. The ability of proteins containing the pleckstrin homology domain, to interact with βγ subunits provides an obvious mechanism to sequester a pool of βγ subunits that is independent of G-protein α subunits [38]. The ability of 3E7 to cause dissociation of βγ even in the absence of guanine nucleotide exchange leaves open the possibility that other proteins could achieve a similar result by interacting with the site recognized by 3E7. Analysis of chimeric α subunits have implicated the carboxyl-terminal region as being of critical importance for receptor coupling [40]. Thus we consider less likely the possibility that activated receptors cause dissociation of βγ from α via the region recognized by 3E7. Therefore we favor the notion that if this region were indeed used to bring about dissociation of G_o, it would involve other proteins, and not heptahelical receptors. Our results obtained with 3E7 suggest that proteins capable of targeting the N-terminal segment of α_o (and by inference, other α subunits) may trigger G-protein dissociation in the absence of nucleotide exchange.

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